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Participation of cathepsin L on bone resorption

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The proteinase responsible for bone collagen degradation in osteo-resorption was examined. The bone pit formation induced by parathyroid hormone (PTH) was markedly suppressed by leupeptin, E-64 and cystatin A, while no inhibition was observed by CA-074, a specific inhibitor of cathepsin B. Pig leucocyte cysteine proteinase inhibitor (PLCPI), a specific inhibitor of cathepsin L, and chymostatin, a selective inhibitor of cathepsin L, completely inhibited the pit formation. Cathepsin L activity in osteoclasts was much higher than the other cathepsin activities. Serum calcium in rats placed on a low calcium diet was decreased by treatment of E-64 or cystatin A, but not by CA-074. These findings suggest that cathepsin L is the main proteinase responsible for bone collagen degradation.

Cathepsin L; E-64; CA-074; Cystatin A; Pig leucocyte cysteine proteinase inhibitor; Bone resorption

1. INTRODUCTION

The osteoclasts are well known to be mainly responsible for the degradation of the organic matrix (mainly collagen fibers) during the process of bone resorption [1–4]. Lysosomal proteinases, possibly a certain cysteine proteinase, in osteoclasts are thought to play an important role in the osseous collagenolysis [2,4–8]. Cathepsins such as cathepsins B, L and N have been shown to be able to efficiently degrade collagen at acidic pH [9–12]. A comparison of collagenolytic activity of these cathepsins has indicated that cathepsin L is particularly strong [4,13,14]. Vaes and his colleagues have shown that there is no correlation between the resorption of cultured mouse bone cells and their secretion of collagenase, while good correlations exist between bone resorption and the excretion of cathepsin B induced by parathyroid hormone (PTH) [6,15]. Furthermore, they also demonstrated that several inhibitors of cysteine proteinases, such as leupeptin, antipain, tosyl-lysyl chloromethane (Tos-Lys-CH_2Cl), benzyl-oxycarbonyl-phenylalanyl-alanyl-diazomethane (Z-Phe-Ala-CHN_2) and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), markedly inhibited the resorption induced by PTH or heparin in cultured mouse bone cells [4–6]. When intraperitoneal injection of E-64 or leupeptin was given to rats, their serum calcium levels and urinary excretion of hydroxyproline significantly decreased 3 and 6 h after administration of the drug [6]. E-64 and leupeptin [16–20] are very powerful inhibitors of all kinds of cysteine proteinases including calpains. These facts suggest the possibility that among lysosomal collagenolytic cysteine proteinases, in particular cathepsins B and L in osteoclasts may play a major role in the bone resorption. However, the particular proteinase responsible for the degradation of the bone collagen has not been decided yet.

This paper reports the proteinase responsible for bone collagen degradation in osteoclastic bone resorption. We investigated the inhibition of bone resorption by various type of inhibitors, such as, pig leucocyte cysteine proteinase inhibitor (PLCPI) [21], a specific inhibitor of cathepsin L, CA-074 [22], a specific inhibitor of cathepsin B, and other inhibitors of cysteine proteinases.

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Abbreviations: Z, benzyloxycarbonyl; MCA, methylcoumarylamide; BCA, bicinchoninic acid; OCPC, *o*-cresolphthalein complexone; E-64-a, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-4-aminobutylamide; CA-074, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline. *Enzymes*: cathepsin B, EC 3.4.22.1; cathepsin L, EC 3.4.22.15.

2. EXPERIMENTAL

2.1. *Materials*

Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA were purchased from the Peptide Institute (Osaka, Japan). E-64-a and CA-074 were kindly supplied from Taisho Pharmaceutical Co., Saitama, Japan. PLCPI and recombinant cystatin A were prepared as described previously [21,23]. Leupeptin, chymostatin, pepstatin and pronase E were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was from Gibco (Grand Island, NY), and human

PTH(1–34) was from Peninsula Laboratories Inc. (Belmond, CA). All other chemicals were of analytical grade. Rat liver cathepsins L and B were purified according to the published procedure [22,24,25]. Sprague–Dawley rats (1–2 days old) used for preparation of unfractionated bone cells, and male Wistar rats (80–100 g) were obtained from Charles River, Japan. Ivory slices (150 μm thick) were punched out as circles 6 mm in diameter, sonicated, and sterilized in 75% ethanol. Low calcium diet (Ca: 0.02–0.03%), a slightly modified Diet-11, was purchased from Nippon Clea (Osaka, Japan). OCPC calcium assay kit was from Wako Pure Chemicals Co. (Osaka, Japan).

2.2. *Methods*

2.2.1. Enzyme assay

Cathepsin activities were measured with Z-Phe-Arg-MCA at pH 5.5 for cathepsin L and Z-Arg-Arg-MCA at pH 6.0 for cathepsin B as substrates respectively by the method of Barrett and Kirschke [26]. Inhibitors were preincubated with enzyme and 8 mM cysteine hydrochloride for 3 min and then reactions were started by addition of the substrates. The activity of each enzyme was adjusted to 0.5 units (one unit expresses as the production of 1 μmol methylcoumarylamine per min from 20 μM of substrate at 37°C). The fluorescence of 7-amino-4-methylcoumarin liberated from the substrate was monitored by a Hitachi fluorescence spectrometer.

2.2.2. Assay of bone resorption

Bone resorption was assessed according to the method of McSheehy and Chambers [27] with certain modifications. Unfractionated bone cells were prepared from tibiae, femora and humeri of 1–2 days old Sprague–Dawley rats. The bones were dissected free of soft tissues in iced tissue culture medium (pH 7.2) [alpha-minimum essential medium (α-MEM) (Flow Laboratories, McLean, VA) supplemented with 100 IU per ml benzylpenicillin] containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES). For preparation of bone cells the long bones were minced with scissors in the same medium. The suspension was then triturated with a wide bored plastic pipette. Two hundred μl of the suspension (1×10^6 cells per ml) was added to each well of 96-well plate containing an ivory slice, and the cells were incubated at 37°C in a CO_2 incubator (5% CO_2:95% air) for 2 h. The slices were then rinsed with α-MEM and transferred to fresh medium containing 10% FBS, various concentrations of inhibitors and 50 nM of PTH, but not containing HEPES, and were incubated for 72 h. The cells were removed by vigorously washing with distilled water, and then the slices were stained with 0.1% Toluidine blue for 5 min. The total area of pits was measured under a light microscope to detect possible osteoclastic bone resorption.

2.2.3. Cysteine proteinase activities in rat osteoclasts

Rat osteoclasts were purified according to the method of Tezuka et al. [28]. After purification, 90% of cells were multinucleate and had tartrate-resistant acid phosphatase activity. The cell suspension in PBS was adjusted to a concentration of 7×10^3 osteoclasts per ml. The cells were then destroyed by sonication, and after centrifugation, the cysteine proteinase activities were assayed as described above. Cathepsins L and B were measured by assay in the presence and absence of 1×10^6 M of CA-074 and cathepsin B was calculated as the CA-074-sensitive Z-Phe-Arg-MCA hydrolyzing activity; the rest of the activity was regarded as cathepsin L activity. Protein concentration in a supernatant was determined by the BCA method (Pierce Chemical. Co., Rockford, IL) with bovine serum albumin as a standard.

2.2.4. In vivo effects of cysteine proteinase inhibitor on serum calcium

Male Wistar rats (80–100 g) received low calcium diet for one week and then starved overnight. E-64-a and CA-074 at doses of 3 and 6 mg per 100 g body weight, respectively, and cystatin A at a dose of 8 mg per 100 mg body weight were injected intraperitoneally to these rats. Blood samples were collected at various times from tail vein under ether anesthesia after the administration of drugs to determine serum calcium concentrations. Serum calcium was measured with OCPC assay reagent by the method of Conerty and Briggs [29].

3. RESULTS AND DISCUSSION

The effects of cysteine proteinase inhibitors on pit formation induced by PTH were investigated, and suppression of pit formation by various proteinase inhibitors were compared in the relation with the inhibition profiles of cathepsins L and B activities shown in Table I. The number of resorptive pits was stimulated by 50 nM of PTH to 3 times more than that of spontaneous group. E-64 and leupeptin markedly inhibited the resorption enhanced by PTH in the range of 1×10^{-8} to 1×10^{-6} M, and also cystatin A [30–32] which strongly inhibits both cathepsins B and L, but does not inhibit calpain, exhibited in the dose-dependent manner at concentrations ranging from 1×10^{-8} to 1×10^{-6} M, indicating that calpain does not participate in the bone resorption. Leupeptin is known to inhibit not only thiol proteinases but also some serine proteinases. However, aprotinin and soya-bean trypsin inhibitor, specific inhibitors of serine proteinases, demonstrated no inhibition on the bone pit formation [4,33]. Therefore, trypsin type serine proteinases are not involved in the bone resorption.

Delaissé et al. reported that PTH-induced excretion of cathepsin B in cultures of embryonic mouse calvaria

Table I

Effects of proteinase inhibitors on the activities of cathepsin L and B, and pit formation

Inhibitor	Concentration (M)	Relative activity (%)		
		Cathepsin L	Cathepsin B	Bone resorption
Control	–	100.0	100.0	100.0 ± 19.4
Blank	–	–	–	31.2 ± 9.81[†a]
Leupeptin	1×10^{-8}	57.2	73.4	42.6 ± 8.2[a]
	1×10^{-7}	8.3	14.4	45.1 ± 14.6
	1×10^{-6}	3.2	3.5	28.0 ± 12.2[b]
E-64	1×10^{-8}	72.8	56.2	50.5 ± 12.9[a]
	1×10^{-7}	37.2	3.4	40.9 ± 13.3[a]
	1×10^{-6}	10.3	1.7	46.2 ± 8.6[a]
CA-074	1×10^{-7}	100.2	2.2	94.2 ± 26.9
	1×10^{-6}	98.3	1.0	73.1 ± 29.7
Cystatin A	1×10^{-8}	22.4	97.2	41.9 ± 17.6[a]
	1×10^{-7}	9.8	44.7	31.2 ± 9.7[a]
	1×10^{-6}	3.1	4.3	17.6 ± 5.4[b]
Chymostatin	1×10^{-6}	9.8	101.3	5.9 ± 0.1[b]
PLCPI	1×10^{-8}	94.4	114.3	73.1 ± 7.5[a]
	1×10^{-7}	43.0	122.2	40.9 ± 1.7[a]
	1×10^{-6}	4.7	124.8	0.1 ± 0.1[b]
Pepstatin	1×10^{-5}	103.3	104.7	104.4 ± 8.8

Enzyme assay: each value indicates the mean of 3 observations. Bone resorption: †Sponaneous (without PTH) bone resorption. Concentration of PTH was 50 nM. Each value indicates the mean ± S.E.M. of 5–6 observations.

[a]$P < 0.05$; [b]$P < 0.01$; significant difference from control group (Student's *t*-test).

was closely correlated with the extent of bone resorption [6]. Therefore, to confirm the role of cathepsin B on bone resorption, we examined the effect of CA-074, a specific inhibitor of cathepsin B, on pit formation. The CA-074 did not inhibit pit formation, suggesting that cathepsin B did not participate in the osteoclastic bone resorption.

Recently, Maciewicz et al. reported that collagens are degraded more extensively by cathepsin L than by other collagenolytic cathepsins [13]. Dalaissé et al. also found cathepsin B, cathepsin L and a cathepsin L-like 70 kDa proteinase in a homogenate of mouse calvaria, showed collagenolytic activity [14]. In addition, in the present study chymostatin which inhibits cathepsin L but not cathepsin B, perfectly inhibited the resorption at a concentration of 1×10^{-6} M. The PLCPI isolated from pig leucocytes [21], a specific inhibitor of cathepsin L, suppressed the formation of pits induced by PTH in a concentration range of 1×10^{-8} to 1×10^{-6} M in a dose-dependent manner. PLCPI, cystatin A and chymostatin at concentration of 1×10^{-6} M inhibited not only PTH-induced pit formation, but also spontaneous pit formation. These inhibition analyses clearly suggest that cathepsin L or cathepsin L-like proteinase is the main proteinase responsible for bone resorption. Moreover, it is possible to speculate that the endogenous cathepsin L inhibitors such as PLCPI and cystatins may participate in the natural regulation of bone resorption phenomenon in physiological and pathological conditions. On the other hand, pepstatin, a specific inhibitor of aspartic proteinase, did not show any inhibitory effect on the pit formation.

With respect to the distribution of cathepsin L in osteoclasts, immunohistochemically cathepsin L is detected not only in lysosomes [34] but also in extracellular resorption lacunae [35]. The contents of various cysteine proteinases in osteoclasts were determined. Z-Phe- Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA hydrolytic activities by the purified rat osteoclasts were assayed as the corresponding activities to cathepsin L plus B, cathepsin B and cathepsin H, respectively. As shown in Table II, Z-Phe-Arg-MCA hydrolytic activity for cathepsin L plus B was found to be the highest, this activity being about 4 times higher than Z-Arg-Arg-MCA hydrolytic activity. The CA-074 inhibited only the part of cathepsin B activity in Z-Phe-Arg-MCA hydrolytic activity, and virtually inhibited Z-Arg-Arg-MCA hydrolytic activity. Thus, the Z-Phe-Arg-MCA hydrolytic activity in the presence of CA-074 corresponds to the cathepsin L activity. On the other hand, Arg-MCA hydrolytic activity for cathepsin H was very weak. Therefore, osteoclasts contain large amounts of cathepsin L or cathepsin L-like proteinase, and this fact also supports the important role of collagenolytic activity of cathepsin L on bone resorption.

Table II

Cysteine proteinase activities in rat osteoclasts

Inhibitor	Cysteine proteinase activities		
	Z-Phe-Arg-MCA (μU/mg)	Z-Arg-Arg-MCA (μU/mg)	Arg-MCA (μU/mg)
None	245.3	60.3	12.7
CA-074†	192.7	4.2	–

Total protein in an extract of rat osteoclasts was 8.7 μg/ml. †Concentration of CA-074 was 1×10^{-6} M.

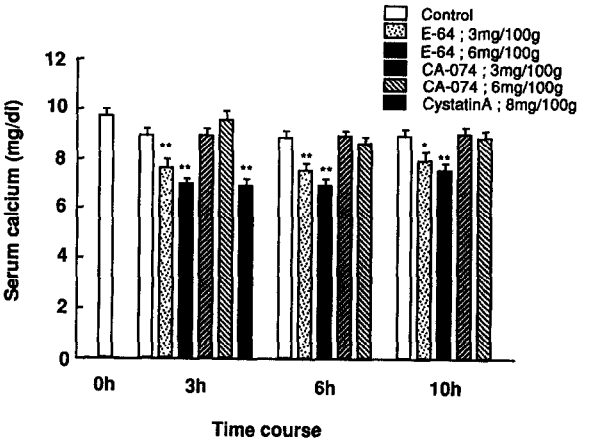

Fig. 1. Effects of E-64, CA-074 and cystatin A on serum calcium levels in low calcium dietary rats. Each value indicates the mean ± S.E.M. of 5 observations. $^{**}P < 0.01$, $^{*}P < 0.05$; significant difference from control group (Student's *t*-test).

Moreover, the results of the pit formation in vitro reflex directly affects the serum calcium level derived from bone resorption in vivo. We examined the in vivo effects of E-64, cystatin A and CA-074 on serum calcium in low calcium dietary rats, and the results are shown in Fig. 1. The injection of E-64 at doses of 3 and 6 mg/100 g body weight to low calcium dietary rats resulted in significant decreases in the serum calcium 3 and 6 h after the injection of the drug in a dose-dependent manner. Furthermore, cystatin A at a dose of 8 mg/100 g body weight significantly decreased calcium level in serum. When the cysteine proteinase activities in the extracts of femur bone were measured one hour after injection of E-64 or cystatin A were measured, all cysteine proteinases in femur bone are inhibited effectively. While, CA-074 did not affect the serum calcium level, although cathepsin B activity in the bone extract was specifically inhibited. From these results, it was reconfirmed in the in vivo system that the cathepsin L, rather than cathepsin B, play a central role in bone collagen degradation.

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